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Cell transplantation therapy for a rat model of secondary lymphedema

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ABSTRACT

Background: Although lymphedema is a progressive and lifelong condition, substantial advances in therapeutic intervention are limited. The development of a novel therapy for lymphedema is urgent for those patients suffering from it. The aim of this study was to investigate the usefulness of a new cell transplantation therapy in the rat tail model of secondary lymphedema.

Materials and methods: We prepared two cell sources, human dermal microvascular endothelial cells (HDMECs) and lymphatic endothelial cells (LECs), which were collected from the resected normal dermis of patients with breast cancer. After the animal model of secondary lymphedema of the nude rats' tails was established, phosphate-buffered saline, purified LECs, or unpurified HDMECs were injected in the rats' tails five times for more than 14 d. The evaluations were performed by measuring the circumference, fluorescence lymphography, and histologic analysis of the rats' tails between each group.

Results: The isolated cells by the simple immunomagnetic sorting from HDMECs were positive for a pan-endothelial marker (CD31) and lymphatic-specific markers (podoplanin, lymphatic vessel endothelial hyaluronan receptor-1 [LYVE-1], and prospero homebox 1 [Prox-1]), and were considered to be LECs. In the cell transplantation group, which was injected with human LECs, the circumference, lymphatic flow, and thickness of the skin of the rat tail became thinner than the groups injected with unpurified HDMECs or phosphate-buffered saline. Immunohistochemistry of the rat tails showed that the number of own lymphatic vessels was increased in the purified LEC transplantation group compared with the other groups. Furthermore, in the LEC transplantation group, some vessels were immunopositive for human-podoplanin or -LYVE-1 and the areas adjacent to the vessels were rat-podoplanin or -LYVE-1 immunopositive.

Conclusions: Our findings indicate that cell transplantation therapy using human LECs improved the secondary lymphedema in the nude rat tail. This therapeutic strategy may merit clinical investigation in patients with lymphedema.

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1. Introduction

The lymphatic vasculature functions to maintain tissue fluid homeostasis by transporting interstitial fluids and proteins to the blood circulation, intestinal lipid absorption, and controls the immune response *via* the transport of lymphocytes and antigen-presenting cells to the lymph nodes [1,2]. Lymphatic vascular insufficiency because of the abnormal vessel development or damaged lymphatic vessels leads to regional swelling known as lymphedema. Although primary lymphedema is a lymphatic malformation developed by the inherited genetic damages, secondary lymphedema is caused by the obstruction or disruption because of malignancy, radiation therapy, surgery, infection, and inflammation [3].

Lymphedema is usually a progressive and lifelong condition for which no curative treatment exists. Lymphedema generally requires combined conservative treatments, currently based on drug therapy, physiotherapy, compression garments, and occasionally surgery [4,5]. Recently, animal models of primary and secondary lymphedema have been treated with viral gene-transfer vectors [6,7] or plasmid DNA that encodes lymphangiogenic growth factors [8–10], or by the transplantation of both lymph nodes and growth factors [11–13]. However, substantial advances in these therapies against lymphedema are limited, and it is possible that the treatments have some side effects such as concomitant blood vessel growth [14,15]. Thus, the development of the other novel therapies for lymphedema is urgent for those patients suffering from it.

Hou *et al.* reported that autologous cell transplantation therapy using bone marrow stromal cells was effective for the treatment of breast cancer–related lymphedema [16]. This therapy seemed to mimic the angiogenesis therapy using peripheral blood mononuclear cells, for implantation into ischemic limbs with ulcers [17,18]. In secondary lymphedema, the lymphatic defect plays a role in the pathogenesis of edema, and therefore lymphatic endothelial cells (LECs) may be also important. It has been reported that LECs could be collected from the dermis by extracting dermal microvessels and sorting them with flow cytometry or immunomagnetic isolation [19–21].

In this study, we investigated the usefulness of a novel cell transplantation therapy in the rat tail model of secondary lymphedema. At first, we prepared the cell sources, human dermal microvascular endothelial cells (HDMECs) and LECs, by an easy and simple method. Next, an animal model of lymphedema of the nude rat tail was established, and HDMECs or LECs were transplanted into this model. We present the improvement in the LEC-transplanted group, suggesting that this new therapeutic strategy using the autologous transplantation may have a possibility of meriting clinical investigation in patients with lymphedema.

2. Materials and methods

2.1. *Culture of human dermal LECs*

2.1.1. *Primary culture of lymphatic microvascular endothelial cells*

This study was approved by the Ethics Committee of Shiga University of Medical Science. Normal full-thickness skin was obtained from female patients undergoing a mastectomy after the written informed consent was obtained. HDMECs were isolated and cultured according to methods previously described [19,21,22] with slight modifications. After the dermis was enzymatically separated and scraped, the cell suspension was seeded onto fibronectin-coated dishes and cultured in endothelial cell growth medium (EGM-2MV; Lonza Group Ltd, Basel, Switzerland) as primary cultures of HDMECs with media exchanges every 2–3 d. At passage 1, the LECs were purified by magnetic-activated cell sorting (MACS; Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions with some modifications [20,23]. After the cell suspension from the primary cultures was incubated with a mouse antihuman podoplanin monoclonal antibody (AngioBio Co, Del Mar, CA) and a goat anti-mouse IgG MicroBeads (Miltenyi Biotec) sequentially, the LECs with the MicroBeads attached were separated magnetically using a MiniMACS separator and cultured in the EGM-2MV on collagen-coated dishes. At subconfluence, a second immunomagnetic purification step was performed as described previously.

On the contrary, HDMECs through the separator, which were not immunomagnetically selected, were cultured in Roswell Park Memorial Institute (RPMI) medium on collagen-coated dishes as unpurified HDMECs.

2.1.2. *Immunofluorescence staining (immunocytochemistry)*

Human LECs (hLECs) or unpurified HDMECs cultured in Lab-Tek chamber slides (Nagel Nunc International, Rochester, NY) were immunostained with the primary antibodies, including a mouse antihuman CD31 monoclonal antibody (AngioBio Co), a mouse antihuman podoplanin monoclonal antibody, a goat antihuman lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), or a rabbit antihuman prospero homebox 1 (Prox-1) polyclonal antibody (ProteinTech Group, Inc, Campbell Park Dr, Chicago), followed by incubation with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Propidium iodide or 4′,6-diamidino-2-phenylindole in the Vectashield mounting medium (Vector Laboratories, Inc, Burlingame, CA) was used for the counterstaining of the nuclei.

The unpurified HDMECs were immunostained with mouse antihuman podoplanin monoclonal antibody, followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibodies.

2.2. *Cell transplantation into the rat tail model of secondary lymphedema*

All protocols for the animal model were approved by the Committee on Research Center for Animal Life Science, Shiga University of Medical Science. A rat model of secondary lymphedema was created as previously described with slight modifications [24]. We used 8-wk-old nude rats (F344/N rnu/rnu; CLEA Japan, Inc, Tokyo, Japan). All experimental rats were fed and housed at Research Center for Animal Life Science, Shiga University of Medical Science, following the previously mentioned guidelines. Nude Rats were anesthetized using pentobarbital sodium (40 mg/kg) delivered by intraperitoneal

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